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# Ecotropic Murine Leukemia Virus Receptor Is Physically Associated with Caveolin and Membrane Rafts

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We used a Sindbis virus expression system to stably express a chimeric ecotropic murine leukemia virus (MLV) receptor gene, CAT1, fused to green fluorescent protein (gfp) in BHK cells. The chimeric gene was expressed on the cell surface and functioned as an MLV receptor. Using gfp as an epitope tag, we found that CAT1 cross-immunoprecipitated with caveolin, a cellular protein associated non-clathrin-coated endocytic vesicles. Biochemical studies showed that CAT1 copurified with caveolin in a detergent-insoluble membrane fraction that forms cholesterol-rich "rafts" on the cell surface. Disruption of rafts by methyl-β-cyclodextrin, a drug that extracts cholesterol, reduced susceptibility to MLV without decreasing surface CAT1. The results indicate that association of the MLV receptor with cholesterol-rich rafts is important for an early step in virus infection.

*Key Words:* MLV receptor; CAT1; rafts; caveolin.

## INTRODUCTION

The cellular receptor for ecotropic murine leukemia virus (MLV) is a transmembrane protein, CAT1, the normal function of which is to transport cationic amino acids into cells (Kim *et al.,* 1991 and Wang *et al.,* 1991). At the first step of infection, MLV particles bind CAT1 via the virus envelope glycoprotein. Fusion between viral and cell membranes appears to occur at the plasma membrane in some cells, while in other cells virus particles may undergo endocytosis prior to fusion since infection is inhibited by alkalinizing agents that block low-pH-induced fusion with other viruses (McClure *et al.,* 1990). In HeLa cells, which can be made susceptible to MLV by introducing the CAT1 receptor, virus entry was inferred to utilize a non-clathrin endosome pathway (Lee *et al.,* 1999) since entry was not inhibited by mutations in dynamin, a protein involved in clathrin-mediated endocytosis.

A second pathway of endocytosis, independent of clathrin, involves caveolae, small plasma membrane invaginations detectable by electron microscopy. The main structural protein of caveolae is caveolin, a membrane protein that binds cholesterol and associates with a detergent-insoluble membrane fraction that is believed to form small, phase-separated microdomains ("rafts") on the cell surface. CAT1 and caveolin were reported to colocalize on the surface of pulmonary endothelial cells using antisera to extracellular loop peptides of CAT1 (McDonald *et al.,* 1997).

Detailed biochemical studies of CAT1 have been hampered by difficulty in generating antisera to CAT1 that work well in immunoprecipitation and Western blot. Two groups recently reported that CAT1-gfp, a chimeric protein with CAT1 at the amino terminus and green fluorescent protein at the carboxyl terminus, retained its function as an MLV receptor while providing a gfp epitope tag for immunodetection of CAT1. The chimeras also made it possible to observe functional CAT1gfp in living cells (Lee *et al.,* 1999 and Masuda *et al.,* 1999).

We have been studying CAT1 function using Sindbis virus expression vectors (Kazachkov *et al.,* 2000). We report here that, using a stable, Sindbis virus vector to express CAT1gfp in BHK cells, antibodies to gfp cross-immunoprecipitate caveolin, and vice versa. Further, as expected for a caveolin-associated protein, CAT1gfp copurifies with detergent-insoluble, cholesterol-rich rafts (Simons and Ikonen, 1995). Methyl-β-cyclodextrin, a drug that extracts cholesterol and disrupts rafts (Keller and Simons, 1998), reduced susceptibility to MLV without significantly reducing cell-surface expression of CAT1. These results show that localization of the MLV receptor in cholesterol-rich rafts is important for MLV entry. Several other virus envelope or receptor proteins are raft associated or cholesterol sensitive, suggesting that caveolae and/or cholesterol play important roles in virus fusion.

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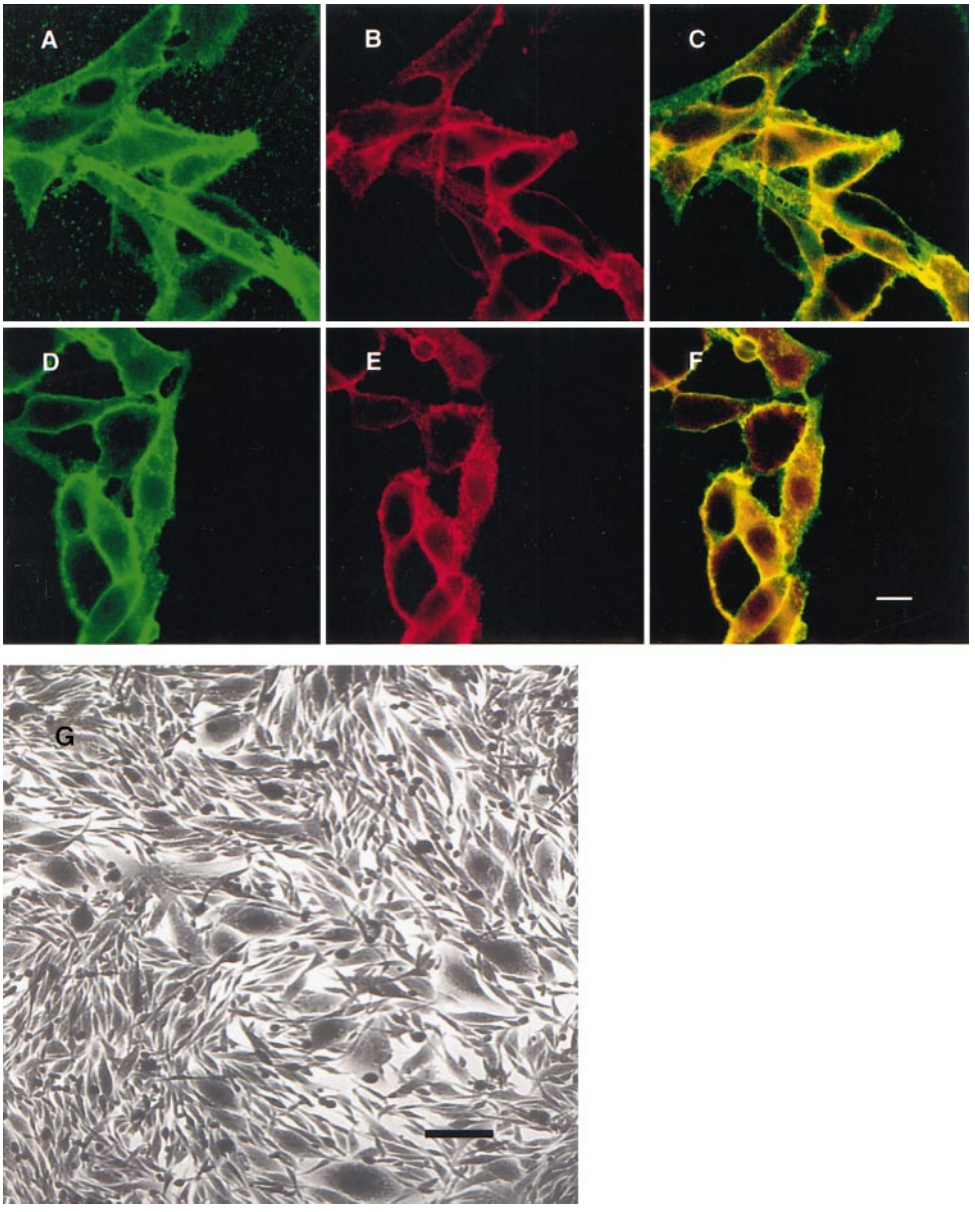


FIG. 1. (A–F) Confocal images of BHKCATgfp cells stained with Texas Red anti-caveolin before (A–C) or after (D–F) treatment with methyl-$\beta$-cyclodextrin. (A and D) gfp signal in green; (B and E) caveolin signal in red; (C and F) overlay in yellow. (G) Transmitted light image of BHKCATgfp cells cocultured with BHK cells expressing MLV envelope, stained to show numerous syncytia. The scale bars are 25 $\mu$m in (A–F) and 100 $\mu$m in (G).

## RESULTS

*Intracellular localization of mCAT1gfp.* We inserted a mouse CAT1-gfp fusion gene downstream of the subgenomic promotor in the noncytopathic Sindbis vector pSINrep19 (Agapov *et al.,* 1998). BHK cells were electroporated with RNA transcribed *in vitro* from this vector and then selected with puromycin. Confocal imaging showed that surviving cells expressed gfp largely on the plasma membrane (Fig. 1A) as expected since CAT1 is a multimembrane-spanning protein. Similar results were previously reported using this and a related CAT1-gfp fusion gene in DNA expression vectors (Lee *et al.,* 1999; Masuda *et al.,* 1999). In some cells, the surface labeling was punctate, as previously reported using anti-CAT1 peptide antisera (Woodard *et al.,* 1994). In addition, many cells contained intracellular fluorescence in the perinuclear region, which colocalized with antibodies to the Golgi marker $\gamma$-adaptin (data not shown). This is expected since wild-type CAT1 and CAT1-gfp are glycosylated in the Golgi before transport to the cell surface. The pattern of gfp fluorescence was different in cells electroporated with a pSINrep19 vector encoding gfp not fused to CAT1; in the latter cells, gfp fluorescence was diffuse in the cytoplasm without membrane accumulation (not shown). For simplicity, we will refer to the BHK cells

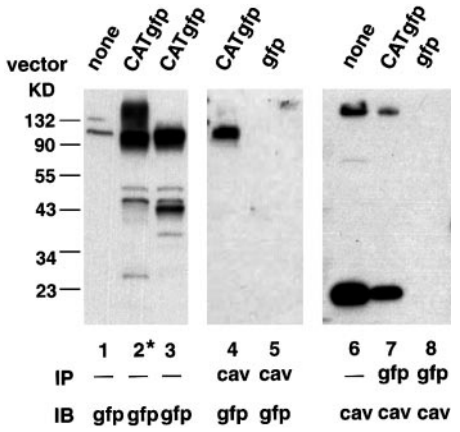


FIG. 2. Cross-immunoprecipitation of CAT1gfp, and caveolin. BHK cells containing pSINrep19CAT1gfp, pSINrep19/GFP, or no vector were lysed and analyzed by SDS–PAGE and immunoblotting directly or after immunoprecipitation. IP refers to antibody used for immunoprecipitation: none (lanes 1–3, 6), monoclonal anti-caveolin (lanes 4 and 5), monoclonal anti-gfp (lanes 7 and 8). IB refers to antibody used to probe immunoblot: polyclonal anti-gfp (lanes 1–5), polyclonal anti-caveolin (lanes 6–8). *The lysate in this lane was boiled in gel loading buffer while other samples were heated to 70°C (see text).

containing pSINrep19CATgfp as BHKCATgfp cells and the BHK cells containing pSINrep19gfp as BHKgfp cells.

Since CAT1 was reported to colocalize with caveolin using anti-CAT1 peptide antisera (McDonald *et al.,* 1997), we compared the intracellular localization of CAT1gfp in our cells with that of caveolin, using an anti-caveolin monoclonal antibody with a Texas Red-conjugated secondary antibody (Fig. 1B). As shown by the yellow color in the overlay image (Fig. 1C), the two proteins were both expressed on the cell surface and colocalized in a somewhat patchy distribution. This pattern is consistent with interaction between CAT1gfp and caveolin but would also be seen if the two proteins were both associated with the plasma membrane. In some cells, caveolin and CAT1gfp were also detected together in peri-nuclear structures that may represent the *trans*-Golgi network. This is not unexpected since caveolin and CAT1 both cycle through the Golgi apparatus (Conrad *et al.,* 1995).

*CAT1 coimmunoprecipitates with caveolin.* To investigate the possible association of CAT1 with caveolin by a different method, we immunoprecipitated caveolin from BHKCATgfp cells and analyzed the immunoprecipitate by Western blot for CAT1gfp. BHKgfp cells were used as a negative control. Anti-gfp antibody detected a major species of ~100–120 kDa in BHKCATgfp cells (Fig. 2, lanes 2 and 3) that was absent in BHK cells (Fig. 2, lane 1). The ~100- to 120-kDa size is consistent with glycosylated CAT1gfp. Others have shown that treatment with the deglycosylating enzyme PNGase F converts this species to one of ~95 kDa, the size expected for nonglycosylated CATgfp (Masuda *et al.,* 1999). Boiling the lysate apparently caused some CAT1gfp to aggregate into a higher molecular weight species (Fig. 2, lane 2 *vs* lane 3), as has been reported for other membrane proteins (Sagne *et al.,* 1996). In some experiments using stored lysates, we also detected minor 40- to 50-kDa species of CAT1gfp that probably represent degradation products. As further controls, the anti-gfp antibody detected the expected ~ 27-kDa gfp in BHKgfp cells that comigrated with purified gfp obtained from Clontech (not shown).

The ~100- to 120-kDa CAT1gfp fusion protein was detected in caveolin immunoprecipitates from BHKCATgfp cells (Fig. 2, lane 4). As a negative control, anti-caveolin antibodies did not co-immunoprecipitate gfp from BHKgfp cells (Fig. 2, lane 5).

Anti-caveolin antibody detected monomeric caveolin (~ 23 kDa) as well as high-molecular-weight, oligomeric caveolin (Monier *et al.,* 1995) in lysates of BHK cells (Fig. 2, lane 6). These caveolin species were also present in gfp immunoprecipitates from BHKCATgfp cells (Fig. 2, lane 7) but not BHKgfp cells (Fig. 2, lane 8).

Cross-immunoprecipitation was detected when cells were lysed with either RIPA buffer or mammalian protein extraction reagent (Pierce Co.), an extraction buffer advertised to minimize disruption of protein–protein interactions. In parallel experiments, we did not detect clathrin in anti-gfp immunoprecipitates (data not shown).

*CAT1gfp copurifies with rafts.* If CAT1 is physically associated with caveolin, a portion of CAT1gfp would be expected to purify with rafts. Rafts proteins are defined operationally by their insolubility in 1% Triton X-100 at 4°C and low density in sucrose gradients (Simons and Ikonen, 1995). We extracted BHKCATgfp cells with 1% Triton X-100 at 4°C and loaded the extract on the bottom of a sucrose gradient. After centrifugation, a milky band could be seen at the position of the interface between the 5 and 30% sucrose layers corresponding to detergent insoluble glycolipids. Proteins from each fraction were analyzed by SDS–PAGE and Western blotting with anti-gfp and anti-caveolin antibodies. Densitometric scanning showed that 47% of the 23-kDa form of caveolin floated to fraction 2, near the top of the gradient (Fig. 3B), as expected for a raft protein. Similarly, 39% of CAT1gfp floated to fraction 2 (Fig. 3A). It is not surprising that portions of caveolin and CATgfp remained at the bottom of the gradient since raft association is incomplete for other, well-documented raft proteins (Scheiffele *et al.,* 1997). As a negative control, we examined the distribution of VSV-Ggfp, a fusion of gfp to the VSV-G membrane protein that does not associate with rafts (Scheiffele *et al.,* 1999). In BHKVSV-Ggfp cells, none of the gfp fusion protein was found in the upper portion of the gradient (Fig. 3C). Similarly, all of the gfp in cells expressing gfp alone (BHKgfp cells) was found at the bottom of the gradient (data not shown).

Rafts are disrupted by depleting cells of cholesterol (Scheiffele *et al.,* 1997). The drug methyl-$\beta$-cyclodextrin is reported to extract up to 70% of cholesterol from BHK

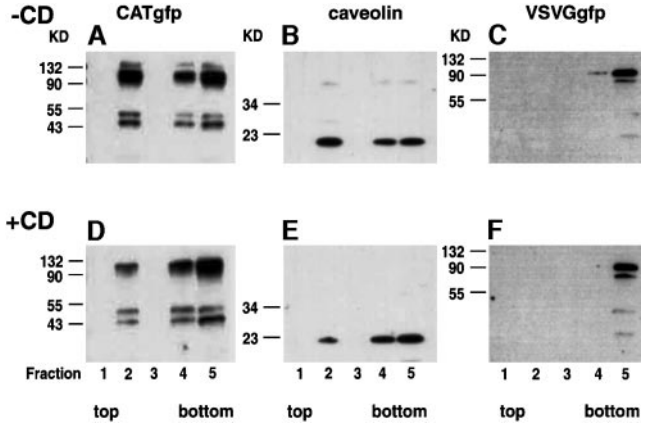


FIG. 3. Sucrose gradiant analysis of detergent insoluble proteins. BHKCATgfp cells (A, B, D, and E) or BHKVSV-Ggfp cells (C and F) were lysed with Triton X-100 at 4°C. Lysates were separated in sucrose gradients, and analyzed by immunoblot with anti-gfp (A, C, D, and F) or anti-caveolin (B, and E). Bottom panels show results for cells treated with methyl-$\beta$-cyclodextrin (+CD); top panels, cells not treated with methyl-$\beta$-cyclodextrin (-CD).

cells after incubation for 30 min in 10 mM methyl-$\beta$-cyclodextrin (Keller and Simons, 1998). This treatment disrupted rafts in BHKCATgfp cells as indicated by the fact that it reduced the proportion of caveolin in fraction 2 from 47 to 7% (Fig. 3E). Similarly, methyl-$\beta$-cyclodextrin reduced the portion of CATgfp in fraction 2 from 39 to 11% (Fig. 3D). As expected, methyl-$\beta$-cyclodextrin had no effect on the migration of VSV-Ggfp (Figs. 3C and 3F) and plain gfp (data not shown).

*CAT1gfp functions as an MLV receptor.* It was important to verify that the CAT1gfp fusion protein in BHKCATgfp cells functioned as an MLV receptor. One measure of functionality of cell-surface CAT1 is its ability to form syncytia with cells expressing MLV envelope. MLV envelope becomes fusogenic after a posttranslational cleavage of its cytoplasmic tail, which is normally mediated by viral protease (Schultz and Rein, 1985). To achieve stable expression of the fusogenic form of envelope in the absence of viral protease, we transfected BHK cells with a vector encoding *neo*$^r$ plus an ecotropic MLV envelope gene containing a stop codon at the cytoplasmic tail cleavage site (Kazachkov *et al.,* 2000). When these envelope-expressing BHK cells were cocultivated with BHKCATgfp cells, large syncytia formed, confirming that CAT1gfp was present on the surface in a form that could bind MLV env and fuse membranes (Fig. 1D). No syncytia formed when BHKCATgfp cells were cocultivated with BHK cells not expressing MLV envelope (Kazachkov *et al.,* 2000; and data not shown).

To test directly for MLV receptor function, we used a MoMLV vector encoding alkaline phosphatase as a marker gene (Miller and Rosman, 1989). Normal BHK cells are not susceptible to MLV because the hamster CAT1 homolog does not function as an MLV receptor (Wilson and Eiden, 1991). Our MLV-alkaline phosphatase virus stock had a titer of $5.3 \times 10^5$/ml in NIH3T3 cells, $1.8 \times 10^5$/ml in BHKCATgfp cells, and <50/ml in plain BHK cells (no alkaline phosphatase cells detected after infection with 20 $\mu$l of virus stock). This shows that the CAT1gfp fusion gene expressed by pSINrep19 provided functional receptor for MLV.

*Cholesterol depletion reduces susceptibility to MLV infection but not cell-surface CAT1.* To see if raft association of CAT1 was important for virus entry, we treated BHKCATgfp cells with 10 mM methyl-$\beta$-cyclodextrin for 30 min prior to challenge with MLV. This treatment reduced susceptibility to MLV ~14-fold (Fig. 4A, middle). As a control for possible toxicity of methyl-$\beta$-cyclodextrin, we treated BHKCATgfp cells with methyl-$\beta$-cyclodextrin for 30 min immediately after virus absorption; this treatment had almost no effect on susceptibility to MLV (Fig. 4A, right). As an additional control, we infected methyl-$\beta$-cyclodextrin-treated cells with an MLV vector pseudotyped by the VSV envelope glycoprotein (VSV-G) since the latter is not raft associated and VSV is not inhibited by cholesterol depletion. Methyl-$\beta$-cyclodextrin had no effect on the VSV-G pseudotype. Similar results were obtained with NIH3T3 cells, which express the natural CAT1 gene (Table 1). To see if methyl-$\beta$-cyclodextrin reduced surface expression of CAT1, we biotinylated surface proteins on BHKCATgfp cells, precipitated cell lysates with streptavidin–agarose, and analyzed the precipitates by Western blot using anti-gfp antibody. The amount of cell-surface CAT1gfp was essentially unchanged (84% of control) after methyl-$\beta$-cyclodextrin treatment (Fig. 4B). Fluorescence imaging of BHKCATgfp cells also showed no obvious effect of methyl-$\beta$-cyclodextrin on surface expression of CATgfp or caveolin (Figs. 1D–1F, 4C, and 4D). These results show that cholesterol depletion inhibits a step that occurs within 1 h of exposure to MLV, most likely virus entry, while leaving receptor on the cell surface.

## DISCUSSION

We used a recently described, stable Sindbis virus vector for expression of functional CAT1gfp chimeric protein in BHK cells (Agapov *et al.,* 1998). One advantage of this system over transient, high-expression Sindbis vectors is that it leads to moderate levels of expressed protein without apparent toxicity, reducing the possibility of artifacts. Another advantage of stable expression is that it allows testing for infection by MLV. The BHKCATgfp cells expressed CAT1gfp on the plasma membrane (Fig. 1) and, as a result, became susceptible to MLV.

Using gfp as an epitope tag, we found that CAT1 was physically associated with caveolin as demonstrated by coimmunoprecipitation of caveolin with antibody to gfp and vice versa in BHKCATgfp cells but not in BHKgfp cells (Fig. 2). Further evidence for association was provided by the finding that a portion of CAT1gfp was raft

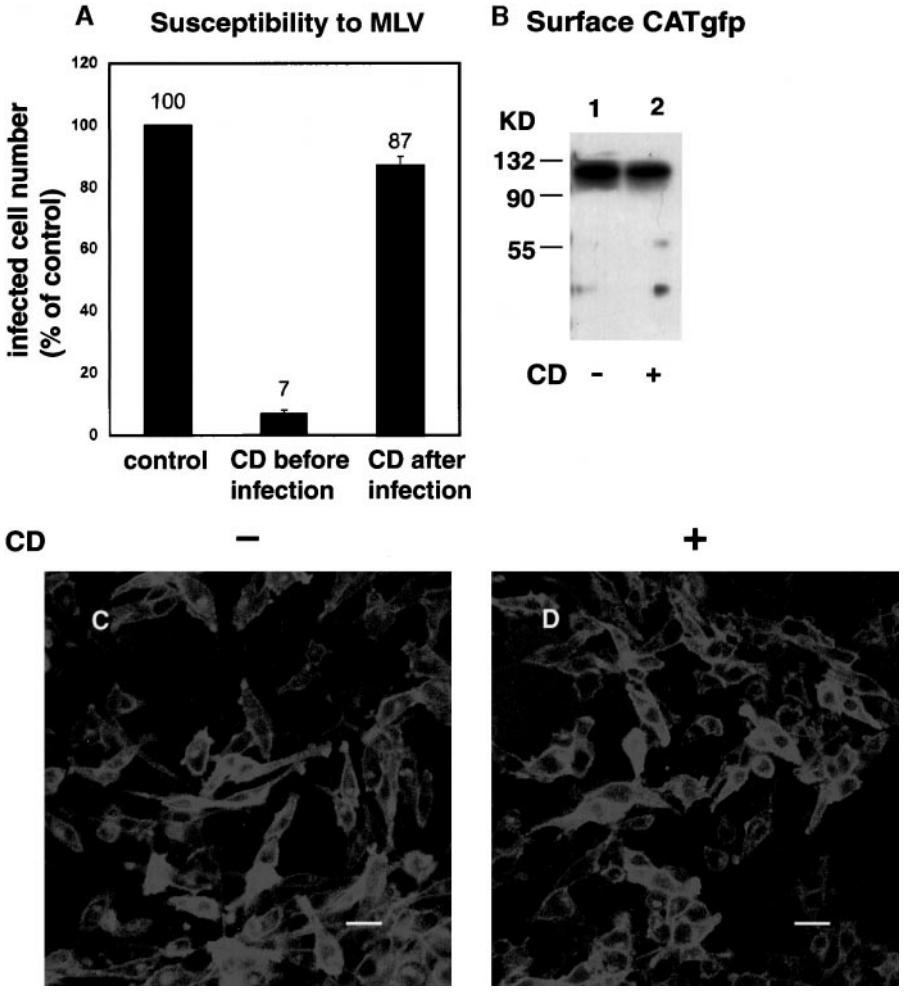


FIG. 4. Effect of methyl-$\beta$-cyclodextrin on susceptibility to MLV, and surface CAT1gfp. (A) BHKCATgfp cells were treated with methyl-$\beta$-cyclodextrin for 30 min just before, or after, infection with alkaline phosphatase-MLV vector. Adherent virus was inactivated after 1 h incubation at 37°C by acid wash. Infected cells were counted 1 day later after staining for alkaline phosphatase. Control cells (not exposed to methyl-$\beta$-cyclodextrin) had ~ 400 alkaline phosphatase-positve cells. Results are the average of two experiments, each done in duplicate. (B) Western blot analysis of surface CAT1gfp. BHKCATgfp cells were treated with sulfosuccinimidyl-6-(biotinamido)hexanoate to label surface proteins. Biotinylated proteins were precipitated with avidin-agarose and precipitates analyzed by SDS–PAGE-immunoblot with anti-gfp antibody. Cells in lane 2 were treated with methyl-$\beta$-cyclodextrin; lane 1, not treated with methyl-$\beta$-cyclodextrin. Confocal images of BHKCATgfp cells untreated (C) or treated with 10 mM methyl-$\beta$-cyclodextrin for 30 min (D). The scale bars are 25 $\mu$m in (C) and (D).

associated, comigrating with caveolin in discontinuous sucrose gradients (Fig. 3).

Since rafts are dissociated by removal of cholesterol, we used the cholesterol-binding agent methyl-$\beta$-cyclodextrin to see if raft association was important for virus receptor function. Methyl-$\beta$-cyclodextrin reduced the portion of caveolin and CAT1gfp that migrated in the raft fraction (Fig. 3) and decreased susceptibility to MLV by ~95% (Fig. 4A and Table 1). Decreased susceptibility to MLV was not a toxic effect because methyl-$\beta$-cyclodextrin did not inhibit MLV when given immediately after, rather than before, virus absorption or when the MLV vector was pseudotyped by VSV-G (Fig. 4A and Table 1). Further, methyl-$\beta$-cyclodextrin treatment did not decrease cell-surface CAT1gfp (Fig. 4B) as it is reported not to downregulate other raft-associated proteins (Green *et al.,* 1999), leading to the hypothesis that membrane cholesterol/raft association of CAT1 is functionally important for MLV entry.

There are two possibly related steps in MLV entry that methyl-$\beta$-cyclodextrin might inhibit. One is endocytosis, particularly if mediated via caveolae. While MLV is reported to fuse at the plasma membrane of most cells, the evidence against endocytosis is that agents that alkalinize lysosomes do not reduce susceptibility to MLV; but this would not rule out internalization pathways not involving acidification. Methyl-$\beta$- cyclodextrin could also inhibit membrane fusion. Fusion is a multistep process with intermediates that have highly curved membrane configurations (Chernomordik *et al.,* 1999). Agents that change spontaneous membrane curvature affect the energy barrier of such intermediates and can promote or

TABLE 1

Effect of Methyl-β-Cyclodextrin on Susceptibility to MLV Vectors Pseudotyped by MLV Env or VSV Env

| Cells | Pseudotype | Cyclodextrin | No. of alk phos- or β-gal-positive cells | Reduction (%) | *P* value |
|---|---|---|---|---|---|
| BHKCATgfp | MLV env | − | 312 ± 14 | | |
| BHKCATgfp | MLV env | + | 19 ± 2 | 94 | <0.0003 |
| BHKCATgfp | VSV env | − | 610 ± 25 | | |
| BHKCATgfp | VSV env | + | 599 ± 22 | 2 | ns |
| NIH3T3 | MLV env | − | 467 ± 41 | | |
| NIH3T3 | MLV env | + | 46 ± 7 | 90 | <0.001 |
| NIH3T3 | VSV env | − | 275 ± 19 | | |
| NIH3T3 | VSV env | + | 254 ± 19 | 8 | ns |

*Note.* Titration done in triplicate. p value calculated by student *t* test. ns, not significant ($p > 0.05$). Values are means ± SD.

inhibit fusion (Chernomordik *et al.,* 1995). The lipid composition of rafts, rich in cholesterol and glycosphingolipids, affects membrane fluidity and curvature. Theoretical models show that patches of membrane with different chemical composition, such as rafts, tend to bud or endocytose depending on their size and edge energy (Julicher and Lipowsky, 1996). Fission and fusion may also be triggered by enzymes that modify lipid composition at the neck of membrane constrictions (Schmidt *et al.,* 1999). Inhibition of MLV entry by removal of cholesterol is likely related to fundamental mechanisms of membrane fusion.

Rafts and cholesterol appear to play important roles in several viruses other than MLV. SV40 induces a signal that promotes its entry into caveolae (Chen and Norkin, 1999). Measles virus, influenza virus, and HIV most likely bud from rafts (Manie *et al.,* 2000, Scheiffele *et al.,* 1999, Aloia *et al.,* 1993; Nguyen and Hildreth, 2000). Influenza virus HA, NA, and M1 proteins have been shown to associate with rafts (Scheiffele *et al.,* 1997; Zhang *et al.,* 2000). The CD4 receptor for HIV is a raft protein (Millan *et al.,* 1999), and HIV fusion is promoted by glycosphingolipids (Puri *et al.,* 1998). Cholesterol and glycosphingolipids are necessary for entry and exit of alphaviruses (Lu *et al.,* 1999, Chatterjee *et al.,* 2000).

Rafts could potentiate virus particle formation, budding, and entry by concentrating molecules involved in these processes or, in the case of virus fusion, by transducing signals initiated by virus binding to the cell surface (Chen and Norkin, 1999). Two recent reports suggest that binding of soluble retroviral envelope molecules to surface receptors alters cells so that they become susceptible to otherwise noninfectious viruses (Lavillette *et al.,* 2000; Anderson *et al.,* 2000), consistent with a signaling process.

In summary, we find that the ecotropic MLV receptor CAT1 is physically associated with caveolin in membrane rafts and that disruption of rafts inhibits an early step in MLV infection. This suggests several intriguing possibilities for the role that caveolae and rafts play in virus entry.

## MATERIALS AND METHODS

*Plasmids, tissue culture, and viruses.* pSINrep19 and pSINrep19/GFP were kindly provided by Charles Rice, Washington University, St Louis, MO. To generate a construct that would express mouse CAT1/gfp as a fusion protein, we amplified mCAT1-gfp (Masuda *et al.,* 1999) kindly provided by Michiaki Masuda (School of Medicine, University of Tokyo, Japan) using primers with artificial *Xba*I and *Apa*I sites (underlined): forward primer 5′ CCTACTCTACACGGTCTAGACACCACCACCATGGGCTGCAAAAACCTG, reverse primer 5′ CCATCATCTCACGGGCCCTTTACTTGTACAGCTCGT. The PCR product was digested with *Apa*I, blunted, digested with *Xba*I, and cloned into pSINrep19 cut with *Xba*I and *Pml*I. This vector is designated pSINrep19CAT1gfp. As a control for membrane expression of gfp via a protein that does not associate with rafts, we amplified a VSV-G-gfp chimeric gene (Presley *et al.,* 1997) kindly provided by Jennifer Lipppencott-Schwartz, NIH, Bethesda, MD, and inserted this gene into the *Pml*I site of pSINrep19. RNA was transcribed *in vitro* from Sindbis vectors linearized with *Not*I. Transcribed RNA was electroporated into BHK cells using a Bio-Rad electroporation apparatus set to 800 V, 25 μF. Puromycin (5 μg/ml) was added 24 h later to select for cells that expressed puromycin acetyl transferase encoded in the pSINrep19 vectors.

Baby hamster kidney (BHK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS). BHK cells that stably express a fusogenic form of MLV envelope (wild-type envelope truncated at the normal p12e cleavage site) have been described (Kazachkov *et al.,* 2000).

To assay for susceptibility to MLV, we used a murine retroviral vector encoding alkaline phosphatase between MoMLV LTRs (LAPSN) (Miller and Rosman, 1989), kindly provided by D. A. Miller, University of Washington, Seattle, WA. An NIH3T3 packaging cell line (Miller *et al.,* 1994) expressing MoMLV gag–pol and the LAPSN vector was infected with MoMLV and 0.45 μm-filtered supernatant from these cells was used as stock virus. Alternatively,

cells expressing Mo-MLV gag-pol and a MLV β-gal vector were transfected with a vector encoding VSV-G (Clontech) to make VSV-G pseudotypes. Target cells were infected with serial dilutions of virus in DMEM-1%FBS containing 4 μg/ml Polybrene for 1 h at 37°C. In some experiments residual, adsorbed virus was inactivated by incubating cells in pH 3.0 citrate buffer (40 mM sodium citrate, 10 mM KCl, 135 mM NaCl) for 1 min at room temperature (Kizhatil and Albritton, 1997). Cells were then washed with PBS and medium was changed to DMEM-5%FBS. One day later cells were stained for β-gal or fixed with 3.7% formaldehyde in PBS at room temperature for 5 min, washed three times with PBS, incubated at 60°C for 30 min to inactivate endogenous cell alkaline phosphatase, and stained with 1 mg/ml nitroblue tetrazolium plus 0.1 mg/ml 5bromo-4chloro-3indolyl phosphate in 100 mM Tris–HCl (pH 8.0), 100 mM NaCl, 50 mM $MgCl_2$ at 37°C for several hours. Alkaline-phosphatase-positive cells were counted by light microscopy.

*Immunofluorescence and confocal microscopy.* BHK-CATgfp cells grown on two-well chamber slides were fixed with methanol at −20°C for 10 min or 3% glutaraldehyde for 15 min at room temperature. Nonspecific binding was blocked with 5% donkey serum in PBS for 30 min. Cells were stained with monoclonal antibody to caveolin (Transduction Laboratories, Cat C37120) in 5% donkey serum in PBS for 60 min at room temperature, followed by Texas Red-conjugated donkey anti-mouse antibody, and mounted with Antifade reagent (Bio-Rad). Samples were observed with a Zeiss LSM410 microscope using 488-nm excitation with a 515- to 560-nm bandwidth filter for gfp and 568-nm excitation with a 590-nm long pass filter for Texas Red.

*Immunoprecipitation and Western blot analysis.* Cells grown in T-75 flasks were lysed in 1 ml RIPA buffer (1× PBS, 1% N-P40, 0.1% SDS, 0.5% sodium deoxycholate) or mammalian protein extraction reagent (Pierce Co.) containing 0.1 mg/ml PMSF, 30 μl/ml aprotinin, 1 mM sodium orthovanadate. The lysate was pipetted through 200- μl pipette tips several times and clarified by brief centrifugation at 4°C. For Western blot, aliquots were heated to 70°C or boiled in an equal volume of Laemmli sample buffer, electrophoresed in 10% SDS–PAGE gels, and transferred to PVDF membranes (Millipore). Membranes were probed with monoclonal anti-gfp antibody (Zymed, Cat 33-2600) or polyclonal rabbit anti-caveolin antibody (Transduction Laboratories, Cat C13630), followed by horse radish peroxidase-labeled anti-mouse or anti-rabbit IgG and an HRP substrate (Pierce, Cat 34075). For immunoprecipitation, cell lysates were prepared as for Western blot analysis and incubated with monoclonal anti-gfp antibody (Zymed, Cat 33-2600), or monoclonal anti-caveolin (Transduction Laboratories, Cat C37120) at 4°C for 1 h, followed by overnight incubation at 4°C with protein A–Sepharose. The beads were eluted in Laemmli sample buffer and analyzed by Western blot using polyclonal rabbit anti-gfp (Clontech, Cat 8367-1).

*Raft membrane purification.* Cells cultured in T-75 flasks were lysed with 0.83 ml of 1% Triton-X100 in ice-cold TNE (50 mM Tris–HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 2 mM DTT). The lysate was pipetted through 200 μl pipette tips several times and incubated on ice for 20 min. After adding one volume of 80% sucrose, the mixture was transferred to the bottom of an ultracentrifuge tube and overlaid with 1.67 ml of 30% sucrose followed by 1.67 ml of 5% sucrose. Following centrifugation at 38,000 rpm in a Beckman SW41Ti rotor for 4 h, five 1-ml fractions were collected from the top. Proteins were precipitated with trichloroacetic acid (TCA), resuspended in PBS, and aliquots were analyzed by SDS–PAGE and Western blot using monoclonal anti-gfp and polyclonal anti-caveolin antibodies as described above.

*Methyl-β-cyclodextrin treatment.* Cells were rinsed with PBS, incubated with 10 mM methyl-β-cyclodextrin (Sigma) (5 mM for NIH3T3 cells) in DMEM for 30 min (15 min for NIH3T3 cells), rinsed twice with DMEM, and cultured in DMEM-5% FBS.

*Biotinylation of surface proteins (Schuberth* et al., *1996).* BHKCATgfp cells in six-well plates were washed with PBS and incubated with 1 mg/ml sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce, Cat 21335) in PBS for 1 h at room temperature. Cells lysates were incubated with streptavidin-agarose for 1 h at 4°C, then washed with cell lysis buffer. Proteins were eluted with SDS–PAGE loading buffer and analyzed by Western blot using monoclonal anti-gfp antibody.

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